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Short communication

In ovo treatment with CpG oligodeoxynucleotides decreases colonization of *Salmonella enteriditis* in broiler chickens

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ABSTRACT

Induction of the innate immune response in newly hatched chickens is important for limiting infections with bacteria, such as *Salmonella enterica* serovar *Enteriditis* (SE). CpG oligodeoxynucleotides (CpG-ODN) can stimulate the innate immune response of young chickens. Therefore, we examined the effectiveness of CpG-ODN administered *in ovo* on intestinal colonization by SE and the ability to modulate the function of heterophils in young chickens. Heterophils were isolated from 2-day-old chickens and were stimulated with heat-killed SE (HK-SE) or PMA for oxidative burst and HK-SE or live SE for degranulation assays. CpG-ODN treatment had no effect on heterophil oxidative burst when stimulated with HK-SE or PMA. However, HK-SE and live SE increased degranulation ($P < 0.01$) in heterophils from CpG-ODN-treated birds compared to PBS-treated controls. In a second experiment, chickens were orally infected with SE on day 10 post-hatch and cecal contents were collected 6 days later for assessment of SE intestinal colonization. CpG-ODN treatment reduced SE colonization by greater than 10-fold ($P < 0.001$) compared to PBS-injected control birds. Overall, we show for the first time that CpG-ODN given *in ovo* stimulates innate immune responsiveness of chicken heterophils and increases resistance of young chickens to SE colonization.

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1. Introduction

The Gram-negative bacteria, *Salmonella*, causes almost half of laboratory-confirmed cases of food-borne illness in the U.S. with *Salmonella enterica* serovar *Enteriditis* (SE) comprising the greatest percentage (FoodNet, 2008). One method for controlling human illness is to decrease pathogenic bacteria in the bird during grow out by increasing resistance to infection (Sadeyen et al., 2004; Swaggerty et al., 2004). Increased resistance to bacteria can be induced by administration of immune modulating substances, including unmethylated DNA that is naturally released by bacteria (He et al., 2007).

During infection, the release of DNA containing unmethylated CpG dinucleotide motifs by bacteria act as potent stimulators of the hosts' innate and adaptive immune response (Krieg, 2002). Certain synthetic CpG-containing oligodeoxynucleotides (CpG-ODN) also stimulate the innate and adaptive immune response of vertebrates (He et al., 2003; Klinman, 2004). Mammalian immune cells are activated by binding of CpG-ODN to an intracellular pathogen-associated molecular pattern (PAMP) recognition receptor known as Toll-like receptor (TLR) 9 (Hemmi et al., 2000). Only cells expressing TLR-9 can directly respond to CpG-ODN. In humans, TLR-9 is present in B-cells and plasmacytoid dendritic cells, whereas in mice, TLR-9 is also expressed in monocytes, macrophages, and myeloid dendritic cells (Klinman et al., 2004). An orthologous gene for TLR-9 in avian species has not been found. However, we have and others have shown that avian heterophils, monocytes, and macrophages

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respond to CpG-ODN in a manner similar to mammals, with a requirement for endosomal maturation, increased nitric oxide production, and increased inflammatory cytokines including IL-6 and IL-1β (He et al., 2003, 2006; He and Kogut, 2003; Xie et al., 2003). *In vivo* studies in mammals and birds show CpG-ODN increases antibody production, maturation of APC's, lymphocyte proliferation, production of T_H1 cytokines and chemokines, nitric oxide production, and heterophil degranulation resulting in decreased bacterial, viral, and intracellular parasitic infection (Dalloul et al., 2004; He et al., 2005b, 2007; Klinman, 2004; Linghua et al., 2007; Patel et al., 2008).

CpG-ODN treatment of chickens could provide a safe, cost-effective alternative to antibiotics by increasing resistance to disease-causing pathogens. However, there are only a few studies that have assessed the effect of CpG-ODN *in vivo* in chickens (Dalloul et al., 2004; Linghua et al., 2007; Roh et al., 2006; Vleugels et al., 2002) with even less information regarding the response to bacterial infection (Gomis et al., 2003, 2004, 2007; He et al., 2005b, 2007). To the best of our knowledge, the effect of *in ovo* CpG-ODN treatment on *Salmonella*-infected chickens has not been determined. We hypothesized that *in ovo* administration of CpG-ODN would increase the immune response of young chickens. Therefore, the objective of this study was to evaluate the *Salmonella*-related heterophil function and resistance of broiler chickens following *in ovo* administration of CpG-ODN.

2. Materials and methods

2.1. ODN and reagents

A synthetic CpG-ODN (CpG#17; GTCGTTGTCGTTGTCGTT), previously shown to stimulate nitric oxide production and inflammatory gene expression in an avian macrophage cell line and oxidative burst and degranulation in heterophils (He et al., 2003, 2005a, 2008), was synthesized on a phosphorothioate backbone (TriLink Biotechnologies, CA). CpG#17 was dissolved in sterile PBS at a concentration of 2 mg/mL and stored at −20 °C before use. All reagents used were purchased from Sigma Chemical Co. (MO) unless stated otherwise.

2.2. Experimental animals

Experiments were conducted according to regulations established by USDA animal care and use committee. Fertilized eggs used in this study were obtained from a commercial broiler chicken breeder. Two groups of 110 eggs and two groups of 275 eggs were set in incubators (Jamesway Incubator Company, Inc., Ontario, Canada) on four different days and maintained at wet and dry bulb temperatures of 32.2 and 37.8 °C, respectively. Chickens from all four groups were used for bacterial challenge, but only chickens from the two larger groups were additionally used for oxidative burst and degranulation assays. After 10 days of incubation, the eggs were candled; non-fertile and non-viable eggs were discarded. The viable eggs were returned to the incubator until day 18, at which time they were equally and randomly divided into two *in ovo* treatment groups (described below). Injected eggs were transferred to hatchers (NatureForm, Jacksonville, FL) and maintained under the same temperature and humidity conditions until hatch. At hatch, treatment groups were kept separate in floor pens containing wood shavings, provided supplemental heat, water, and a balanced, unmedicated corn and soybean meal-based chick starter diet *ad libitum* that met or exceeded the levels of critical nutrients recommended by the National Research Council (1994).

2.3. Bacteria

A primary poultry isolate of SE (phage type 13A) from the National Veterinary Services Laboratory (Ames, IA), selected for resistance to novobiocin (NO) and nalidixic acid (NA) in the USDA-ARS facility (College Station, TX) was used. The SE was passed three times into 100 mL of tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) containing 25 μg/mL NO and 20 μg/mL NA (Sigma Chemical Co., St. Louis, MO). Bacteria for cell assays were diluted to 1×10^9 CFU and confirmed as described below. Bacteria were then heat-killed for 5 min at 76 °C and stored at 4 °C until use. The challenge inoculum was cultured overnight at 41 °C, washed, and resuspended in sterile PBS. The viable cell concentration of the challenge dose and cell assay prior to heat-killing was confirmed by colony counts on Xilose Lisina Tergitol-4 (XLT; Difco Laboratories, Detroit, MI) plates containing NO and NA (XLT-NN). Heat-killed SE (HK-SE) was plated on XLT-NN plates to ensure all bacteria had been killed.

2.4. In ovo injection

To simulate commercial hatchery *in ovo* injection practices, treatments were administered to 18-day-old embryos. Approximately 75% of the eggs set were viable at day 18 of embryogenesis. The remaining eggs were then equally divided into PBS and CpG#17 (25 μg per egg) treatment groups. Eggs were injected with 100 μL of PBS or 100 μL of CpG#17 diluted in PBS. Endotoxin-free, cell-culture tested PBS was used to minimize a possible placebo effect. All injections were performed essentially as previously described with the following modification (Sharma and Burmester, 1982). The large end of each egg was wiped with iodine and gently scored with an 18-gauge needle. Treatments were administered into the amnion using a 1-cm^3 tuberculin syringe and a 25-gauge needle equipped with a modified needle guard to limit all injections to a depth of 3 cm. Following *in ovo* treatment to individual embryos, the injection sites were covered with melted paraffin using a cotton swab, and eggs were placed into the hatcher.

2.5. Isolation of heterophils, oxidative burst assay, and degranulation assay

Blood from two biological replicates of 30–35 chickens was collected in EDTA containing tubes and pooled to obtain approximately 65 mL of blood for each treatment group on two separate hatches (four pooled samples per

treatment). Heterophils were isolated from the peripheral blood of uninfected chickens 48 h post-hatch using established isolation techniques (Kogut et al., 1995). Isolated heterophils were quantified and diluted to 1×10^7 cells/mL in RPMI-1640 media. Oxidative burst assay was performed as previously described (He et al., 2007) with the following modifications. Chicken heterophils (1 mL) were unstimulated or stimulated for 60 min with phorbol myristate acetate (PMA, 1 μg/mL) or HK-SE (1×10^8 bacteria/mL). Degranulation assays were carried out essentially as described by He et al. (2007), except cells (1 mL) were unstimulated or stimulated for 60 min with HK-SE (1×10^8 bacteria/mL) or live SE (1×10^8 bacteria/mL). Previous research by our group has shown PMA and SE to stimulate oxidative burst and degranulation assays, respectively (He et al., 2005a; Kogut et al., 1998). Therefore, these stimulants were used as positive controls in their respective assay. HK-SE was the antigen used for *in ovo* treatment and was therefore used in both assays.

2.6. Bacteria challenge and culture

Twenty to 25 chickens from each *in ovo* treatment group were infected orally with 1×10^6 SE on day 10 post-hatch to determine if CpG administration had longer lasting immune protection. Chickens were euthanized and necropsied 6 days post-challenge. PBS-challenged chickens were included as a negative control and remained uninfected throughout the study. The ceca were aseptically removed, 0.25 g of cecal contents was diluted in 2.5 mL of PBS, three 10-fold serial dilutions were made from diluted cecal contents, and 100 μL of the last three dilutions was spread evenly onto XLT-NN plates. Plates were incubated for 24 h at 41 °C, and colony-forming units (CFU) from plates with countable colonies were enumerated and averaged to express $\log_{10}$ CFU of SE per gram of cecal contents. Four separate replicates were conducted with chickens hatched on different dates, with a total of 90 birds per treatment group.

2.7. Statistical analysis

Data from oxidative burst and degranulation assays were analyzed as a split-plot mixed ANOVA using the PROC Mixed procedure of SAS (SAS 9.1.3, 2002). Day of heterophil isolation was modeled as a random factor and sample by *in ovo* treatment was modeled as the error term for assessment of differences between *in ovo* treatments. Specific differences between *in ovo* treatment means were determined within cell treatment (unstimulated or stimulated with HK-SE, SE, or PMA). Log_{10} CFU enumerations were analyzed as a one-way ANOVA, randomized block design with hatch dates as the random factor in Proc Mixed. Tests of differences were determined using a Student's *t*-test and considered significant if $P < 0.05$.

3. Results and discussion

Mammals, fish, and birds recognize synthetic CpG-ODN's that mimic bacterial DNA produced as a result of host infection. *In vivo* studies in chickens show i.m., s.c., and i.p. injections of CpG-ODN's increase immune responsiveness to bacterial and viral infections (Gomis et al., 2003, 2007; He et al., 2005b, 2007; Linghua et al., 2007). The few studies that evaluated the use of CpG-ODN's *in ovo* report birds have increased immunity to *Eimeria*, an intracellular parasite, and to the Gram-negative bacteria, *Escherichia coli* (Dalloul et al., 2004; Gomis et al., 2004). To determine the effectiveness of using CpG-ODN as an immune modulator to limit SE infection in chickens, PBS or CpG#17 were given by *in ovo* injection on day 18 of embryogenesis. Chickens were then assessed for *in vitro* heterophil function and resistance to colonization after SE challenge.

There was an increased oxidative burst in stimulated heterophils compared to unstimulated heterophils from both PBS- and CpG#17-treated birds, indicating that the cells had the potential to respond to invading pathogens. However, comparison of *in ovo* treatment groups revealed CpG#17 treatment did not increase heterophil oxidative burst compared to PBS controls when cells were stimulated with HK-SE or PMA (Fig. 1A). Reactive oxygen species (oxidative burst) generated by activated heterophils aid in killing and removal of bacterial pathogens through the innate immune response (Harmon, 1998). However, avian heterophils do not produce as many bactericidal components as mammalian neutrophils and likely depend more

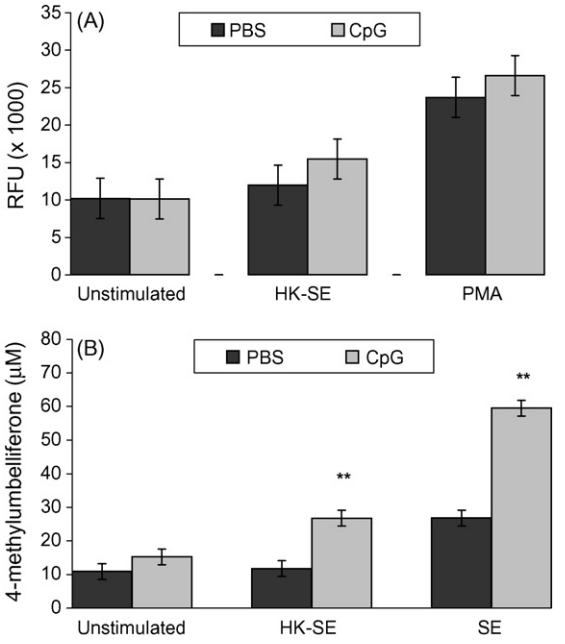

Fig. 1. Effect of *in ovo* treatment (PBS or CpG) on *in vitro* heterophil oxidative burst (A) and degranulation (B). Chicken embryos were treated with either 100 μL of PBS or synthetic CpG oligodeoxynucleotides (CpG#17, 25 μg) on day 18 of embryogenesis. Blood was collected 2-days post-hatch from 30 to 35 birds and was pooled for heterophil isolation. Heterophils for oxidative burst were unstimulated or stimulated with heat-killed *Salmonella enterica* serovar *Enteriditis* (HK-SE, 1×10^8 cells) or PMA and presented as relative fluorescence units (RFU). Degranulation was measured in heterophils left unstimulated or stimulated with HKSE (1×10^8 cells) or live SE (1×10^8 cells). Data represent least-square means and standard errors of four biological replicates. $^{**}P < 0.01$ difference between means of *in ovo* groups treated with the same stimulant.

strongly on oxygen-independent mechanisms for killing of bacteria (Harmon, 1998).

Recognition of bacterial PAMPs through innate immune receptors, such as TLR's, also enable heterophils to release toxic granules that contribute to bactericidal activity (He et al., 2005a; Kogut et al., 2005). In the present study, heterophils from CpG#17-treated birds had increased degranulation compared to PBS controls when the cells were stimulated with HK-SE and live SE (Fig. 1B). Similarly, He et al. (2007) found i.p.-injected CpG#17 given 24 h before sampling to young chickens increases heterophil degranulation with no difference in oxidative burst. The previous study also found that birds given CpG#17 48 and 96 h pre-sampling had increased oxidative burst compared to PBS-injected controls, which is in contrast to our findings that oxidative burst did not change with CpG#17 treatment. The different routes of treatment and age of the birds when treated with CpG#17 may account for the differences observed between studies. Increased heterophil activity is of greater importance during the first 4 days after hatch because chickens are susceptible to SE infection, in part, as a result of immature heterophils (Wells et al., 1998). Therefore, priming of heterophils through *in ovo* treatment may initiate heterophil maturation and a stronger response to SE infection.

Chickens were orally challenged with SE on day 10 post-hatch and cecal contents cultured for enumeration of SE 6 days post-challenge to determine the lasting effects of CpG#17 *in ovo* treatment. CpG#17 treatment ($6.2 \pm 0.24 \log_{10}$ CFU) reduced cecal colonization over 10-fold compared to PBS-treated controls ($4.9 \pm 0.24 \log_{10}$ CFU). Our results are in agreement with Gomis et al. (2003), reporting i.m. injection of CpG-ODN in *E. coli*-infected chickens decreases the presence of systemic bacteria. Decreased coccidial oocyst shedding seen in chickens given an *in ovo* CpG-ODN injection also support our results (Dalloul et al., 2004). In mice, CpG-ODN treatment has been extensively studied *in vivo* and is effective in reducing infections of multiple bacteria, viruses, and parasites (Klinman et al., 2004). The lasting effects of CpG#17 on reduction in bacterial infection may be due to a stronger innate response and also greater antibody production, resulting in a stronger adaptive immune response during infection (Ameiss et al., 2006). As it is still not clear how CpG-ODN binds to and activates chicken cells, the immune modulation caused by CpG-ODN may not be the same in avian and mammalian species. Therefore, further evaluation of the route and location of CpG-ODN administration, CpG-ODN dosage, and how optimization of these factors may vary in chickens with different genetic backgrounds needs to be assessed.

The effects of CpG-ODN treatment on immune parameters of birds infected with bacteria (cytokine expression, nitric oxide production, oxidative burst and degranulation, antibody production, cell proliferation, etc.) and measures of early *in vivo* bacterial resistance (mortality and organ invasion) have been assessed (Ameiss et al., 2006; Gomis et al., 2003, 2004, 2007; He et al., 2005b, 2007; Patel et al., 2008). To the best of our knowledge, this is the first time that the effect of *in ovo* CpG treatment on SE cecal colonization in chickens has been reported. We showed that CpG given *in ovo* increased heterophil function and decreased the presence of SE in the ceca. By limiting cecal colonization in the chicken during production, we can reduce contamination of the carcass during processing, ultimately decreasing the chance of human infection and illness.

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